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The Walter and Eliza Hall Institute of Medical Research

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PROVISIONAL SPECIFICATION

for the invention entitled:

"Novel proteins, their derivatives, homologues and analogues and uses therefor"

The invention is described in the following statement:

NOVEL PROTEINS, THEIR DERIVATIVES, HOMOLOGUES AND ANALOGUES AND USES THEREFOR

Members of the family of suppressors of cytokine signalling (SOCS) proteins contain a central SH2 domain and a C-terminal homology domain we have termed the SOCS box (1,2). The first member of this family was called CIS (cytokine-inducible SH2-containing protein) (3) and was shown to inhibit erythropoietin and interleukin-3 receptor signalling. We cloned SOCS-1 from a retroviral expression library as a cDNA whose constitutive expression inhibited interleukin-6-induced differentiation of M1 cells (1) and it was simultaneously cloned by others as a protein that interacted with activated JAK kinases (JAK-binding protein, JAB) (4) and as a protein with antigenic similarity to STATs (STAT-inducible STAT inhibitor, SSI) (5). The sequence similarity of SOCS-1 and CIS led to the discovery of six additional members of this family (SOCS-2-7) each with an SH2 domain and a C-terminal SOCS box (2,6,7). An additional twelve proteins have been described that contain a C-terminal SOCS box but instead of an SH2 domain they contain different protein-protein interaction domains including WD40, ankyrin repeats, SPRY or small GTPase domains (2).

Following binding to their receptors, many cytokines activate receptor-associated cytoplasmic kinases called JAKs which in turn phosphorylate the receptor cytoplasmic domain and associated signal transducers and activators of transcription (STATs). Phosphorylated STAT dimers translocate to the nucleus and activate transcription of specific genes including those of CIS and some of the SOCS. SOCS proteins then recognize activated signalling molecules (including JAKs and cytokine receptors) through their SH2 and N-terminal domains and inhibit their activity (8,9). Exactly how SOCS proteins inhibit JAK kinase activity and the role of the conserved SOCS box are currently unknown. In the present report, the inventors show that the SOCS box interacts with elongins B and C and through them with the proteasome complex. Targeting of SOCS proteins and their bound activated signalling molecules to the protein degradation pathway explain how SOCS proteins simultaneously terminate a cytokine stimulation cycle and their own inhibitory action so that cells may respond to a second round of stimulation.

Accordingly, the present invention provides a family of adapter proteins which terminate cell signalling by targeting critical molecules for intracellular degradation.

The present invention further provides molecules capable of interacting with the various protein-interaction domains of the SOCS family of proteins.

The present invention further contemplates a method of terminating cytokine stimulation or antagonisting termination of cytokine stimulation.

The present invention is further described by the following non-limiting Figures and Examples.

In the Figures:

Figure 1. Purification of SOCS box-binding proteins from murine myeloid M1 cells. Panel A, SDS-PAGE (14% Novex gel) analysis of affinity column eluates from GST-Sepharose column (lane 1), from GST-SOCS-1-SOCS-box-Sepharose column (lane 2), and from GST-SOCS-3-SOCS-box-Sepharose column (lane 3). The proteins were visualized by Coomassie blue staining. *Arrows* in lane 2 indicate the positions of the two protein bands excised for sequencing analysis by mass spectrometry. The molecular mass markers (in kilodaltons) are shown on the left. Panel B, Western blot analysis of the three affinity column eluates mentioned in panel A by anti-rat elongins B and C antibodies. Anti-rat elongins B and C antibodies (cross react with murine and human elongins B and C) were purchased from Santa Cruz and used as a mixture of 1 ?g/ml of each antibody.

Figure 2. Competition of SOCS-1 SOCS box interaction with elongin C. Biotinylated SOCS-1 SOCS box peptide was immobilized on streptavidin-agarose resin and used to affinity purify interacting proteins from M1 cellular extracts in the presence (+) or absence (-) of 80 mM competing non-biotinylated SOCS box peptides. Proteins were separated by SDS-PAGE on a 4-15% acrylamide gel, and bands were visualized by silver staining. Soluble SOCS box peptides corresponding to SOCS-1, ASB-2 and WSB-2 prevented binding of elongin C to immobilized SOCS-1 SOCS box

Figure 3. Interaction of SOCS-1 with endogenous elongins B and C. Celluar extracts from M1 cells stablly expressing either full-length SOCS-1 or SOCS-1 lacking SOCS box (both proteins were N-terminally FLAG-tagged) were incubated with anti-FLAG antibody M2 resin and bound cellular proteins were eluted from the columns with FLAG peptide as described in Materials and Methods. Lanes 1-3 correspond to column eluates 3 to 5 from M1 cells expressing full-length SOCS-1 and lanes 4-6 correspond to column eluates 3 to 5 from M1 cells expressing SOCS-1 lacking SOCS box (SOCS-1/?SB). The panels from top to bottom correspond to Western blot analyses by anti-FLAG, anti-elongin C, anti-elongin B, and a mixture of anti-elongin B and anti-elongin C, respectively.

Figure 4. Co-transfection of 293T cells with SOCS and elongins B and C.

Figure 5. Effect of LLnL on the endogenous expression of SOCS-3 protein. The murine macrophage-like J774 cells (4 x 10^7) were treated with either DMSO (0.1%) or LLnL (50 μM) for 15 min and then stimulated with 100 ng/ml of murine IL-6 for the indicated times in the presence of DMSO or LLnL during stimulation. The cellular extracts were immunoprecipitated with 1 ?l of a rabbit-anti-SCOS-3 polyclonal antiserum and immune complexes eluted from protein G-Sepharose beads were resolved by SDS-PAGE (13%) under reducing conditions and analysed by Western blot using biotinylated rabbit-anti-SOCS-3.

Figure 6. Model of the interaction of SOCS box-containing proteins with elongins C and B (upper panel) and comparison with the phosphoprotein ubiquitin ligase complex (PULC) assembled by F box –containing proteins (lower panel).

EXAMPLE 1

MATERIALS AND METHODS

SOCS and Elongin Expression Vectors. The cDNAs encoding mouse SOCS-1,SOCS-3, WSB-1, SSB-1, ASB-1, have been described previously (1,2,9). Constructs in pEF-FLAG1 encoding these proteins, with or without the SOCS box, with an N- terminal FLAG epitope tag (DYKDDDDK) were generated by PCR essentially as described (1,9) (found at http:// www.wehi. edu.au/ willson vectors). DNA fragments encoding mouse elongins B and C were amplified using PCR from a 17-day embryo cDNA lambda library (Clontech ML5014t) and were expressed with N-terminal FLAG or myc (DQKLISEEDL) epitope tags, respectively, using the mammalian expression vector pEF-BOS.

Stable and Transient Transfection of Cell Lines. The murine monocytic leukemic cell line, M1, and the 293T human fibroblast cell line were maintained and transfected as described (9).

Preparation of GST and GST-SOCS Box Affinity Resins. DNA fragments encoding the SOCS boxes from mouse SOCS-1 (residues 172-212) and SOCS-3 (residues 186-225) with an N-terminal linker sequence (EGKSSGSGSESKVD) were generated by PCR and cloned into the bacterial expression vector pGEX-2T (10). The GST fusion proteins were purified by affinity chromatography on glutathione Sepharose 4B (Amersham Pharmacia Biotec) and affinity resins were prepared by covalently coupling 1 mg of purified proteins to 1 ml of NHS-activated Sepharose resin (Amersham Pharmacia Biotec). Before use, the affinity resins were washed with elution buffer (0.5% SDS, 50 mM DTT, 50 mM Tris-HCl, pH 8.0 and 150 mM NaCl) and equilibrated in lysis buffer (0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.5 and 100 mM NaCl).

Purification of SOCS Box-Binding Proteins. M1 cells (2×10^{10}) were lysed on ice for 30 min in 100 ml of lysis buffer supplemented with protease inhibitors (Complete Cocktail tablets; Boehringer Mannheim, Mannheim, Germany), 1 mM PMSF, 1 mM Na_3VO_4 and 1 mM NaF. The total cell lysate was centrifuged at 15,000 rpm (SS34 rotor) for 15 min at 4°C and the clarified supernatant pre-incubated with 1 ml of GST-Sepharose resin for 2 h at 4°C. Half the

GST-Sepharose-depleted M1 cell lysate was incubated with 1 ml of GST-SOCS-1 SOCS box- and the other half with GST-SOCS-3 SOCS box-Sepharose resin for 2 h at 4°C. The affinity resins were washed with 40 ml of lysis buffer and then eluted with 8 x 0.5 ml of elution buffer. Eluates were concentrated to ~40 µl ,mixed with 15 µl of 4 x SDS sample buffer containing 0.4 M DTT and resolved on a 14% polyacrylamide gel (Novex). The gel was stained for 5 min with 0.1% Coomassie blue in 50% methanol and destained in 12% methanol and 7% acetic acid.

Protein Identification by Peptide Mass Fingerprinting. Protein bands were excised from the Coomassie blue-stained gel and *in-situ* tryptic digestion was performed as described previously (11). An electrospray ion trap mass spectrometer (LCQ Finnigan MAT, San Jose, CA) coupled on-line to a capillary HPLC (12,13) was used for peptide sequencing. The sequences of individual peptides were identified manually or by using the SEQUEST algorithm (incorporated into the Finnigan-MAT BIOWORKS™ software) to correlate the collision-induced dissociation spectra with amino acid sequences in the OWL protein database (14).

Peptide synthesis and biotinylation. Peptide fragments of murine SOCS-1, WSB-2 and ASB-2 corresponding to the SOCS boxes and five upstream N-terminal residues (2) were synthesized according to the *in situ* neutralization/HBTU activation protocol for Boc solid phase chemistry (15), purified using reverse phase HPLC and the products characterized by electrospray mass spectrometry. A sample of the SOCS-1 SOCS box peptide was post-synthetically biotinylated by treatment with sulfosuccinimidobiotin. Prior to biotinylation, the sidechain of the unique cysteine residue was temporarily protected by oxidation to the peptide disulfide dimer. The SOCS-1 SOCS box peptide contains no lysine residues, thus excess biotinylation reagent was used to completely and specifically modify the the amino terminus. Following biotinylation, the peptide was reduced by treatment with 5 mM DTT. Typically, peptide was coupled to streptavidin-agarose resin (Pierce immunopure; 1-2 mg streptavidin/mL resin) by incubating equal volumes of resin and 1 mg/mL peptide for 1 h, followed by extensive washing.

Competition of SOCS 1 SOCS box/elongin C interaction. M1 cells were lysed as previously described, except at a concentration of 10^9 cells/mL of lysis buffer. Streptavidin-agarose

binding proteins were precleared from lysate by treating overnight at 4°C with streptavidin-agarose resin (100 µL of resin/1 mL lysate). SOCS box peptides (SOCS-1, ASB-2 and WSB-2) were solubilized in water at 10 mg/mL, and aliquots of these, or water alone, were added to 350 µL fractions of cleared lysate, followed by incubation for 3 h at 4°C. These lysates were then added to 30 µL of SOCS-1 SOCS box peptide resin and incubated a further 2 h at 4°C. The resin was extensively washed with lysis buffer and bound proteins were eluted with 20 µL of 4x SDS sample buffer. Proteins were separated by SDS-PAGE on a 4-15% reducing gel.

Detection of SOCS-1 Interaction with Endogenous Elongins. Two litres of M1 cells stably expressing either full-length SOCS-1 or SOCS-1 lacking SOCS box (with N-terminal FLAG epitopes) were grown in DME containing 5% bovine calf serum, 10 µg/ml puromycin and 50 ng/ml murine IL-6. The cells were harvested and incubated in 20 ml of culture media containing 10 µM proteasome-specific inhibitor, *N*-acetyl-L-leucinyl-L-leucinyl-norleucinal (LLnL; Sigma, St. Louis, MO) for 30 min at 37°C. The cells were lysed in 14 ml of lysis buffer supplemented with protease inhibitors (Complete Cocktail tablets), 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and 10 µM LLnL. Total cell lysates were centrifuged at 15,000 rpm (SS34 rotor) for 15 min at 4°C and the clarified supernatants incubated with 0.3 ml of M2 anti-FLAG antibody resin for 3 h at 4°C. Resin was then washed with 10 ml of lysis buffer and the bound proteins were eluted with 6 x 150 µl of 100 µg/ml FLAG peptide in lysis buffer.

IL-6-Induced Expression of Endogenous SOCS-3 Protein. Mouse macrophage-like J774 cells were grown continuously in DME containing 10% bovine calf serum. The cells were washed once in PBS, twice with DME and starved for 1 h in DME containing 0.1% low-endotoxin bovine serum albumin (BSA; Sigma). The proteasome inhibitor LLnL dissolved in dimethyl sulfoxide (DMSO) or DMSO was added to the cells for 15 min and the cells then stimulated with 100 ng/ml of murine IL-6 for the indicated times.

Co-immunoprecipitation and Western analysis Proteins were immunoprecipitated with anti-myc (9E10; WEHI) and protein A-Sepharose or anti-FLAG antibody conjugated to Sepharose (KM5-IC7;WEHI) and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Biorad, Hercules, CA) under reducing conditions. Proteins were

then electrophoretically transferred to PVDF-Plus membranes (Micron Separations Inc. WestBorough, MA) and Western blotted as described (9).

EXAMPLE 2

RESULTS

Although the SOCS box appears to be a modular sequence motif present in at least twenty different proteins it does not appear to be required for inhibition of the JAK/STAT signalling pathway when SOCS proteins are overexpressed (8,9). The inventors reasoned that the SOCS box might play a regulatory role in targeting proteins to particular cell compartments or in controlling the in vivo half-lives of proteins which may become important considerations when SOCS proteins are expressed at physiological levels. The SOCS box domain is unlikely to be large enough to encode catalytic activity and is therefore likely to mediate such effects through protein-protein interactions. Consequently, the ability of the SOCS box to interact with cellular target proteins was investigated.

Isolated SOCS box sequences were used as affinity reagents to identify interacting proteins in cell lysates. Glutathione-S-transferase (GST) fusion proteins containing the SOCS box sequences from SOCS-1 or SOCS-3 were coupled to Sepharose beads and used as affinity resins to bind proteins from M1 cell lysates. After washing the beads, bound proteins were eluted with SDS buffer and electrophoresed on SDS-PAGE gels followed by staining with Coomassie Blue. The most prominent bands seen binding to both GST-SOCS fusion proteins but not the GST control were proteins of 15 and 18 kDa (Fig. 1). These bands were excised from the gel and digested in situ with trypsin. Tryptic peptides were separated by reverse-phase capillary HPLC and the column eluate fed directly onto a electrospray ion trap mass spectrometer. Collision-induced dissociation of the molecular ions was used to determine the amino acid sequences of the tryptic fragments and these were correlated against sequences in the OWL protein data base. The 18 kDa band generated 10 peptides that could be identified as belonging to elongin B and the 15 kDa band generated 5 peptides that could be identified as belonging to elongin C (Table 1). Western blotting of the gels of the same eluates with antibodies against elongins B and C confirmed that both elongins were present in eluates from

beads containing SOCS-1 or SOCS-3-box fusion proteins but not from control GST bands (Fig. 1).

Similar experiments using a biotinylated SOCS-1 SOCS box peptide bound to streptavidin-agarose also resulted in the identification by mass spectrometry of elongins B and C as interacting proteins in M1 cellular extracts. The specificity of this interaction was tested by pre-incubating extracts with unbiotinylated SOCS box peptides prior to addition of the imobilized SOCS-1 SOCS box peptide. As expected, unconjugated SOCS-1 SOCS box peptide competed for this interaction as did SOCS box peptides from, WSB-2 and ASB-2 suggesting that interaction with elongins B and C is a general property of the conserved SOCS box (Fig.2). Interestingly, identical results were obtained whether M1 cells were stimulated with cytokine (IL-6 or LIF) or not.

The inventors next tested the capacity of full-length or SOCS box-deleted SOCS proteins to interact with elongins B and C in M1 cells. M1 cells stably transfected with vectors encoding N-terminally FLAG-tagged full-length SOCS-1 or SOCS-1 lacking a SOCS box (SOCS-1/ΔSB) were lysed, the FLAG-tagged proteins were immunoprecipitated with anti-FLAG M2 antibody beads and the beads were eluted with FLAG peptide. The eluates were electrophoresed on SDS-PAGE gels, transferred to PVDF membranes and Western blotted with anti- FLAG antibodies or antibodies to elongins B and C (Fig. 3). Although full-length SOCS-1 and SOCS-1/ΔSB were expressed at similar levels, only the full-length SOCS-1 protein was associated with bound elongins B and C.

To further confirm the generality of this interaction for other proteins containing a SOCS box, 293T fibroblasts were transfected with N-terminally FLAG-tagged WSB-2 or SSB-1 along with elongin B containing a FLAG epitope or elongin C containing a myc epitope. When elongin C was immunoprecipitated with anti-myc antibodies and the electrophoresed eluates Western blotted with anti-FLAG antibodies, both WSB-2 and SSB-1 were found to co-immunoprecipitate along with elongin B with elongin C (Fig. 4). As with SOCS-1, the interaction of elongins B and C with SSB-1 was dependent on the SOCS box as a truncated form lacking only the SOCS box failed to co-immunoprecipitate with the elongins (Fig. 4).

Because elongins B and C have been proposed to target proteins to proteasomal destruction (16, 17), the inventors tested whether endogenous SOCS proteins are degraded through the proteasomal complex. When the J774 macrophage cell line was stimulated with IL-6, SOCS-3 protein expression was elevated by 30 min, peaked at 60 min and was significantly depleted by 120 and 180 min. In contrast, cells incubated with the proteasomal inhibitor LLnL and stimulated with IL-6 showed a continual increase in SOCS-3 protein levels from 30-180 min (Fig.5) suggesting that the proteasomal complex plays a major role in rapidly degrading SOCS-3 after its induction.

EXAMPLE 3
DISCUSSION

The SOCS proteins were initially defined as cytokine-inducible inhibitors of cytokine signalling and thought of as closing a classical negative feedback loop (1,4,5). Their direct linkage to the JAK/STAT signalling pathway was revealed by the observations that the expression of at least some SOCS proteins were induced by the STAT transcriptional activators, that they bound to activated JAKs and inhibited JAK kinase activity and thereby suppressed activation of STATs and the subsequent ability of STATs to activate transcription of indicator genes (1,4,5,9).

The C-terminal homology domain common to SOCS-1 and CIS was subsequently shown to be conserved in six other SOCS proteins that each also contained a central SH2 domain. In addition, 12 other proteins were shown to contain a C-terminal SOCS box but these proteins did not contain a SH2 domain; instead they contained other protein-protein interaction domains such as WD40, ankyrin repeats, SPRY or small GTPase domains (2).

The present specification has shown that a common role of SOCS boxes from several different classes of proteins is to bind to elongins B and C. The elongin B and C complex has previously been shown to bind to elongin A to form an active transcriptional elongation complex or to the von Hippel Lindau (VHL) tumor suppressor protein (16,17). The sites on elongin A and VHL that interact with elongin C have been mapped and the consensus binding sequence

(T,S,P)LXXX(C,S)XXX(LIV) is also conserved in the N-terminal half of all SOCS boxes (17).

The elongin B/C complex appears to have two distinct roles. When bound to elongin A it acts as a positive transcriptional regulator by increasing the activity of the RNA polymerase II elongation complex (18) but when bound to VHL it acts to suppress the accumulation of hypoxia-inducible mRNAs (19). Initially it was thought that VHL might act as a transcriptional suppressor by sequestering elongins B and C and making them unavailable to interact with elongin A but more recent studies have suggested an alternate mechanism of action. The VHL/elongin B,C complex contains a putative E3 ubiquitin ligase (Cullin-2) that may target VHL-binding proteins to destruction by the proteasome. Cullin-2 appears to interact with elongin C (directly or indirectly) independently of subsequent association with VHL (19). Elongin B also contains a ubiquitin-like (UBL) sequence at its N-terminus (19) in common with several other proteins. One of these (RAD23) has recently been shown to interact directly with proteasomal subunit proteins (Cim3 and Cim5) through its UBL domain leading to an increase of protease activity associated with RAD23 (20). Analysis of the VHL gene in individuals with VHL disease has revealed that the interaction domain with elongin C is commonly mutated and that most affected individuals show a reduced ability of VHL to interact with elongins B and C (21-24). Similarly mutation of the UBL domain in RAD23 in yeast leads to ultraviolet light sensitivity suggesting that it plays an important regulatory role in nucleotide excision repair (20). These observations suggest that coupling of VHL or RAD23 proteins to the proteasome is essential for the correct functioning of these proteins.

Put together with the inventors data on the binding of elongin B/C to the SOCS box these observations suggest a model for the action of SOCS proteins (Fig. 6). As shown previously (9) the N-terminal and SH2 domains of SOCS-1 and SOCS-3, at least, are required for recognition and binding to activated (tyrosine phosphorylated) signal transduction molecules (eg JAKs). The SOCS box brings into this complex elongins B and C and either through direct interactions of the elongin B UBL domain with the proteasome or through associated Cullin-2-induced ubiquitination of substrates and subsequent proteosomal association, the substrate and associated SOCS protein may be destroyed. In this scheme both activated signal transduction molecules and their negative regulators (SOCS proteins) would be destroyed after a cytokine

activation cycle and the cell would be ready to respond again if cytokine is still present.

In overexpression studies, the SOCS box was not required to inhibit cytokine signalling (8,9). This implies that SOCS interaction with its targets is sufficient to inhibit signalling and that the role of the SOCS box interaction with elongins B and C may be primarily to terminate the inhibitory signal by destroying the SOCS protein. The present data indicate that the SOCS box confers protein instability on SOCS-3 in a proteasome-dependent manner. In situations where SOCS proteins are expressed at physiological levels, the ability to degrade SOCS-associated signalling molecules may become important in order to achieve maximal inhibition of cytokine-generated signals.

It was also noted in the present study that intact SOCS proteins bound less well to elongins B and C than did isolated SOCS box peptides (at least for SOCS-1 and SOCS-3). This may suggest that SOCS box availability for interaction with elongins is dependent on conformational changes associated with SOCS protein binding to its activated targets (eg JAKs). Given the efficiency of the proteosomal protein degradation system it may make some sense for SOCS proteins and signal transduction molecules to be destroyed only after they have interacted with their targets.

The model proposed above for the function of the SOCS box has a very strong parallel with components of the phosphoprotein-ubiquitin ligase complex (PULC) that is utilized to control various aspects of the cell cycle (25). In the yeast PULC system, serine phosphorylation of the cyclin-dependent kinase (cdk) inhibitor sicI or G1 cyclins Cln1 and Cln2 leads to their recognition by adaptor proteins such as Cdc4 or Grr1 which contain conserved N-terminal domains called F-Boxes. The F-Box mediates interaction with Skp1, an elongin C homologue, which in turn interacts with E2 and E3 (Cullin homologue) ubiquitin ligases. This results in ubiquitination of the phosphorylated substrates and targeting for proteasomal degradation so that the cell cycle can progress from G1 to S.

Recently, Verdier et al (26) showed that CIS, a member of the SOCS family of proteins, is monoubiquinated and subject to proteasomal degradation. Moreover, they also showed that

inhibitors of the proteasome lead to sustained expression of activated forms of the erythropoietin receptor and STATS following erythropoietin stimulation of UT-7 cells. Similarly, Yu and Burakoff (27) showed that inhibition of the proteasome resulted in sustained activation of the JAK/STAT pathway following interleukin-2 stimulation although neither appeared to be ubiquitinated. The present results provide a mechanism for targeting such proteins to proteasomal degradation via association of signalling molecules with the SOCS/CIS proteins followed by SOCS-box-mediated interaction with elongins B and C. It is possible that this interaction results in ubiquitination of SOCS/CIS and associated molecules (mediated by cullins) or that non-ubiquitinated proteins in the complex are delivered to the proteasome via the UBL sequence in elongin B. Another function of monoubiquitination of receptors is to target them to endocytosis and subsequent degradation by lysozymes rather than the proteasome (28). Consequently coupling of cell signalling molecules to the ubiquitination pathway can lead to termination of the signalling response in several different ways.

The present report has demonstrated that the single conserved domain in 20 structurally diverse proteins (the SOCS box) serves to couple bound proteins to the ubiquitination or proteasomal compartments through interaction with elongins B and C. The SOCS box-containing proteins thus form a family of adapter proteins, analogous to the F-Box-containing proteins, which terminate cell signalling by targeting critical molecules for intracellular degradation.

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Patent Attorneys for the Applicants

Table 1. Tandem mass-spectrometric characterization of elongin B as the 18 kDa band and elongin C as the 15 kDa proteins bound by SOCS box sequences

Protein	Peptide No	Experimental[a] MH^+(Da)	Predicted[a] MH^+(Da)	Sequence[b]	Position in Protein
18 kDa Protein	1	1161.6	1162.3	HKTTIFTDAK	10-19
	2	771.5	772.0	IVEGILK	30-36
	3	1196.3	1196.3	ESSTVFELKR	20-29
	4	927.7	928.2	RIVEGILK	29-36
	5	1664.9	1664.9	IVEGILKRPPEEQR	30-43
	6	2339.6	2339.7	HKTTIFTDAKESSTVFELKR	10-29
	7	1917.8	1918.2	TTIFTDAKESSTVFELKR	12-29
	8	3056.6	3056.3	IEPFSSPPELPDVMKPQDSGGSANEQAVQ	90-118
	9	4075.0	4075.4	ADDTFEALRIEPFSSPPELPDVMKPQDSGGSANEQAVQ	81-118
	10	1066.5	1067.4	MDVFLMIR[c]	1-8
15 kDa Protein	1	1213.2	1213.4	REHALTSGTIK	33-43
	2	1009.6	1010.2	EIPSHVLSK	64-72
	3	1596.5	1596.8	TYGGCEGPDAMYVK[d]	7-20
	4	1501.5	1501.7	LISSDGHEFIVKR	21-33
	5	1159.5	1160.4	VCMYFTYK[d]	73-80
	6	2213.1	2212.4	AMLSGPGQFAENETNEVNFR	44-63

Sequence determination of peptides was performed by tandem mass-spectrometry in an ESI-IT mass spectrometer. Ten tryptic peptides analysed from the 18 kDa-protein corresponded to sequences in rat elongin B(Genbank accession number L42855) and six tryptic peptides analysed from the 15 kDa-protein corresponded to sequences in rat elongin C (Genbank accession number L29259) with mass errors of 0.004 – 0.080%. These peptides covered 68.6% and 66.1% of the elongins B and C sequence, respectively.

(a) Average mass values.

(b) Amino acid sequence is given using the one-letter notation.

(c) N-terminal methionine is acetylated (+42 Da).

(d) Cysteine residue is alkylated with 4-vinyl pyridine during sample preparation (+105 Da).

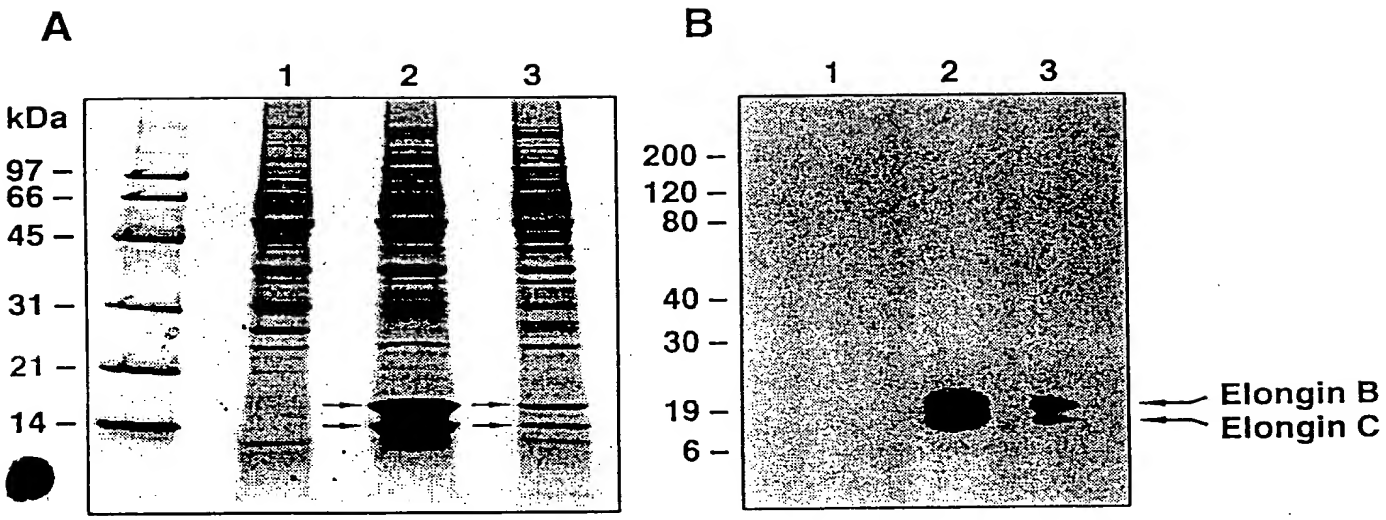

A
1
2
3
kDa
97 –
66 –
45 –
31 –
21 –
14 –
B
1
2
3
200 –
120 –
80 –
40 –
30 –
19 –
6 –
Elongin B
Elongin C

FIG. 1

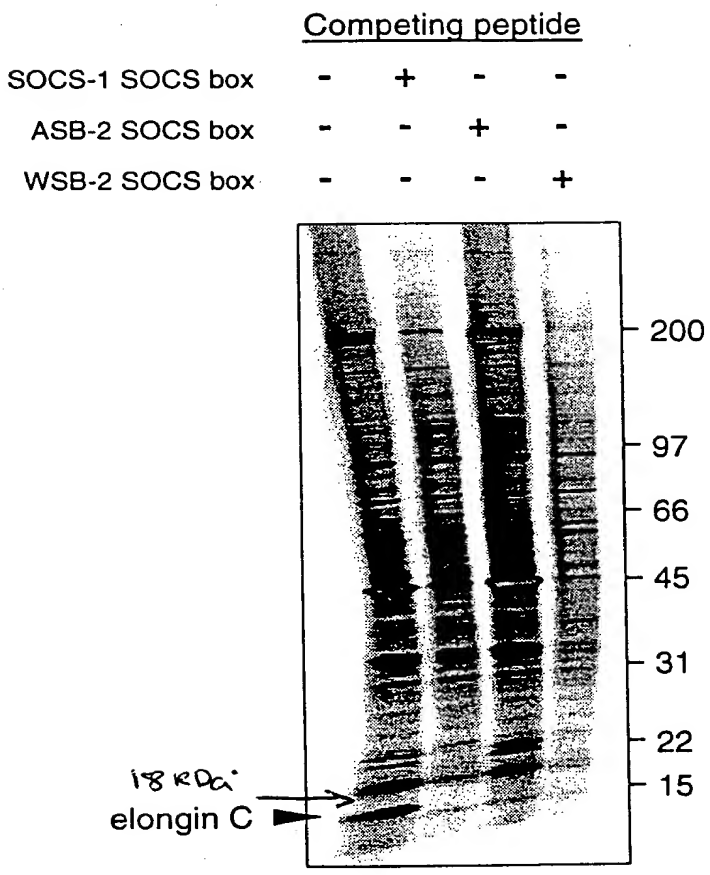

Competing peptide
SOCS-1 SOCS box - + - -
ASB-2 SOCS box - - + -
WSB-2 SOCS box - - - +
200
97
66
45
31
22
15
18 KDa
elongin C

FIG. 2

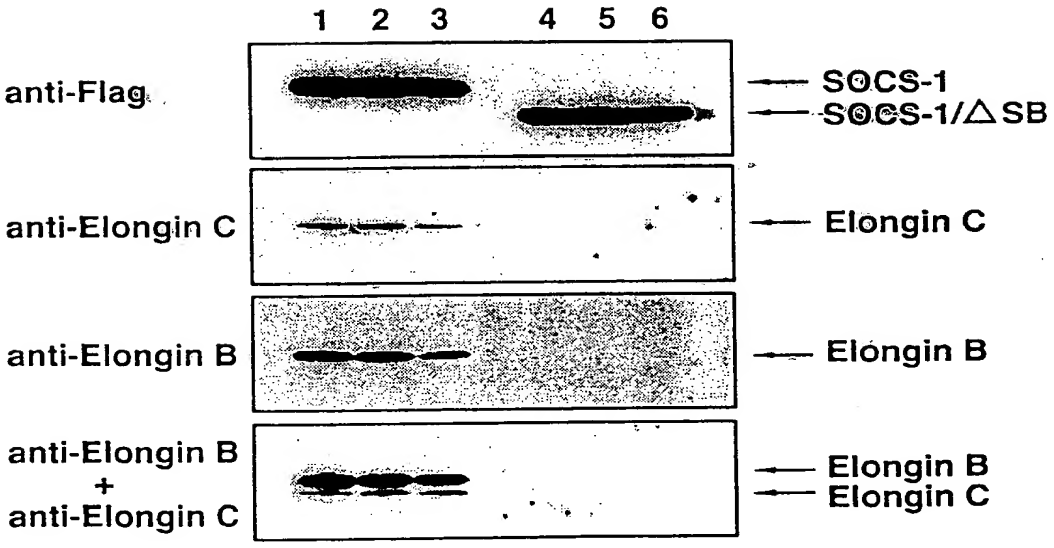
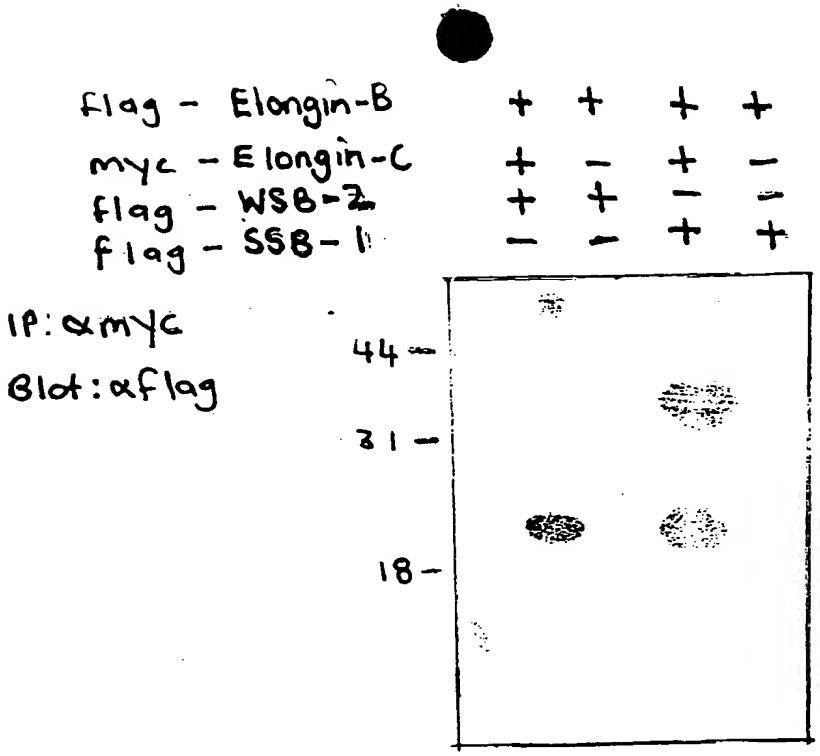

1 2 3 4 5 6
anti-Flag
SOCS-1
SOCS-1/△SB
anti-Elongin C
Elongin C
anti-Elongin B
Elongin B
anti-Elongin B
+
anti-Elongin C
Elongin B
Elongin C

FIG. 3


flag - Elongin-B + + + +
myc - Elongin-C + - + -
flag - WSB-2 + + - -
flag - SSB-1 - - + +
IP: αmyc
Blot: αflag
44
31
18

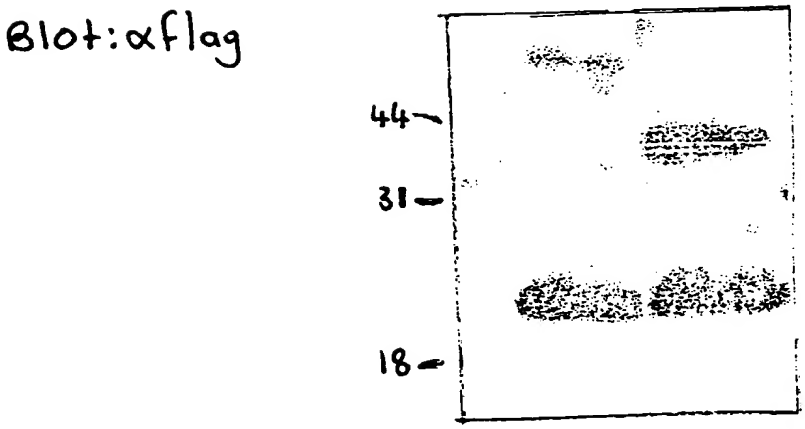

Blot: αflag
44
31
18

FIG. 4

981010

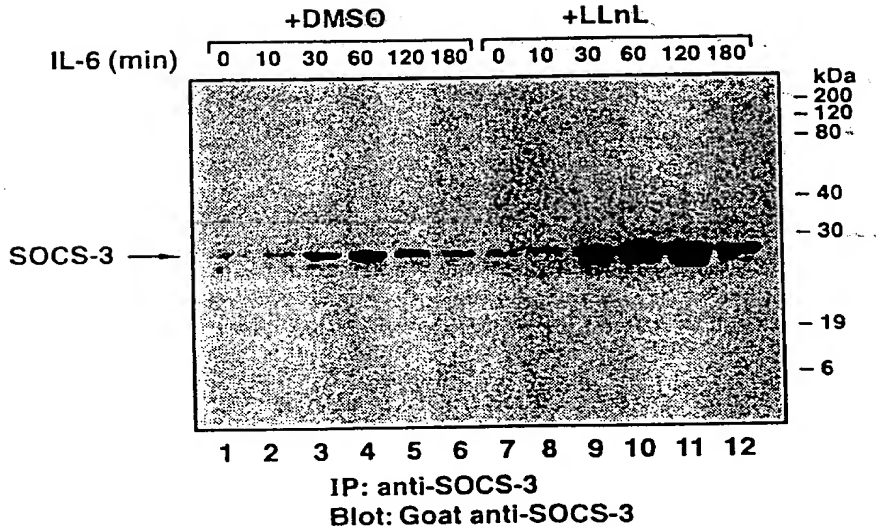
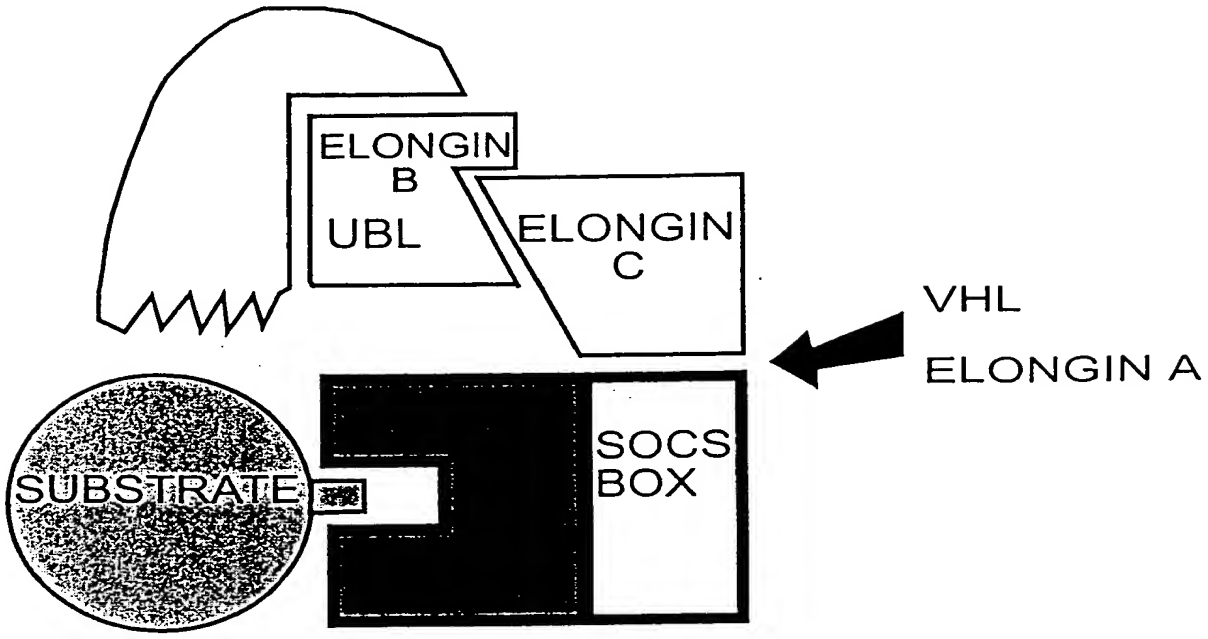

+DMSO
+LLnL
IL-6 (min)
0 10 30 60 120 180 0 10 30 60 120 180
kDa
200
120
80
40
30
19
6
SOCS-3
1 2 3 4 5 6 7 8 9 10 11 12
IP: anti-SOCS-3
Blot: Goat anti-SOCS-3

FIG. 5


PROTEASOME
ELONGIN B
UBL
ELONGIN C
VHL
ELONGIN A
SUBSTRATE
SOCS BOX

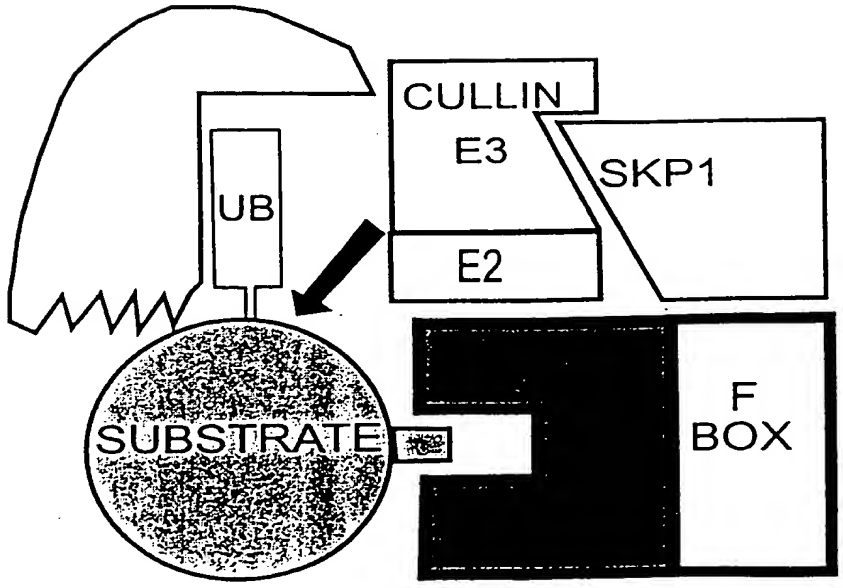

PROTEASOME
CULLIN
E3
SKP1
UB
E2
SUBSTRATE
F BOX

FIG. 6